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# The effect of pulsed electromagnetic fields on the physiologic behaviour of a human astrocytoma cell line

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**Abstract**

We evaluated the effects of 50 Hz pulsed electromagnetic fields (EMFs) with a peak magnetic field of 3 mT on human astrocytoma cells. Our results clearly demonstrate that, after the cells were exposed to EMFs for 24 h, the basal $[Ca^{2+}]_i$ levels increased significantly from 124 ± 51 nM to 200 ± 79 nM. Pretreatment of the cells with 1.2 μM substance P increased the $[Ca^{2+}]_i$ to 555 ± 278 nM, while EMF exposure caused a significant drop in $[Ca^{2+}]_i$ to 327 ± 146 nM. The overall effect of EMFs probably depends on the prevailing $Ca^{2+}$ conditions of the cells. After exposure, the proliferative responses of both normal and substance P-pretreated cells increased slightly from 1.03 to 1.07 and 1.04 to 1.06, respectively. U-373 MG cells spontaneously released about 10 pg/ml of interleukin-6 which was significantly increased after the addition of substance P. Moreover, immediately after EMF exposure and 24 h thereafter, the interleukin-6 levels were more elevated (about 40%) than in controls. On the whole, our data suggest that, by changing the properties of cell membranes, EMFs can influence $Ca^{2+}$ transport processes and hence $Ca^{2+}$ homeostasis. The increased levels of interleukin-6 after 24 h of EMF exposure may confirm the complex connection between $Ca^{2+}$ levels, substance P and the cytokine network. 

*Keywords:* Intracellular calcium; Cell proliferation; Substance P; Caffeine; Cytokine; Interleukin-6

## 1. Introduction

The numerous electromagnetic fields (EMFs) arising from sources in all residential and occupational settings are the direct result of electrical power generation, distribution and utilization [1]. The magnetic fields generated in domestic and commercial environments are higher than those occurring naturally; although they depend on the current flow, they decrease rapidly with distance from the source. These nonionizing radiations of frequencies ranging from 0 to 300 Hz and 0.1 to 100 mT can influence several cellular activities, with unusual dose-response characteristics [2] since the mechanism of the modified biochemical and physiological processes is directly linked to the frequency [3].

EMFs have been implicated as a contributing factor in the incidence of cancer [4] and the studies of Feycthing et al. [5,6] have associated potential hazards with exposure to weak EMFs. Recently studies of carcinogenicity in adults and children have evidenced limited or inadequate evidence that residen-

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tial exposure to low frequency EMFs is carcinogenic [7]. This is also stated by the work of Linet et al. [8] and by the results of UK Childhood Cancer Study Investigators [9].

The contentious nature of this topic is probably due to either the risk assessment methodology [10], or the lack of a generally accepted mechanism to explain how very low frequency EMFs might induce changes in biological systems. Therefore many in vitro experiments, which are easier and more reproducible than in vivo investigations, have been performed. It has been demonstrated that EMFs can activate many cell types and induce the release of cytokines after mitogen challenge [11–14].

Cytokines are low molecular weight proteins that participate in a wide variety of biological responses. Identified primarily by their function in the immune response, they play an important role in establishing and maintaining the normal homeostatic environment of the central nervous system (CNS). The ability of cells in the human CNS to produce cytokines is suggested by the detection of these proteins in cerebrospinal fluid and in the nervous tissue itself during the course of various diseases. In fact cytokines act either directly or indirectly on the regulation of the biological responses of the CNS to injury [15]. There is increasing evidence suggesting that multifunctional cytokines regulate crucial steps in neural development and post-lesion responses. One of the most numerous cell types in the brain is the astrocyte which exhibits immune and inflammatory responses [16] as well as other functions, including neuronal migration, neurite outgrowth, osmotic homeostasis, maintenance of the blood-brain barrier and synaptogenesis. One immune function of astrocytes is the production of interleukin-6 (IL-6). Released within the CNS, this cytokine can act on glia, lymphocytes and neurones, producing several different responses [16]. It has been demonstrated that both human astrocytes and human astrocytoma cell lines are able to produce proinflammatory cytokines [17,18] such as tumour necrosis factor-α (TNF-α) and IL-6. At present, nothing is known about the possible physiological effects of EMFs on astrocytes, although effects on the nervous system are well documented [19–21]. The human astrocytoma cell line U-373 MG, like astrocytes, expresses a single class of high affinity NK-1 receptors coupled to phosphoinositide turnover. These are receptors for substance P which is an 11 amino acid neurotransmitter related to the tachykinin family of neuropeptides [22]. Substance P stimulates the increase of both cytoplasmic $Ca^{2+}$ and IL-6 secretion by U-373 MG cell [18,23,24]. Many types of stress may affect the intracellular free calcium dynamics [25,26] and the flux of $Ca^{2+}$ appears to be modified by specific EMF frequencies [27], although this is not universally accepted [28] and Boorman et al. [29], using both 50 and 60 Hz EMFs exposures in regional facility studies, were unable to evidence any effect on either calcium transients or ornithine decarboxylase activity, or MYC gene expression.

Anyhow, EMFs are capable of generating interactions between cations and ion channel proteins in the membranes [30], while a clustered distribution of intramembrane proteins was also observed by Bersani et al. [31] after EMF exposure. Since $Ca^{2+}$ is involved in the regulation of many cellular functions, the movement of this ion in the context of membranes can explain the diversity and variety of cellular responses observed.

In this study we investigated whether EMFs could promote movements of $Ca^{2+}$, cell proliferation and the eventual production of IL-6 in human astrocytoma U-373 MG cells after exposure to EMFs for 24 h. The same study was also performed after pretreatment of the U-373 MG cells with substance P. Moreover, in order to evaluate whether $Ca^{2+}$ movements were due to release of calcium from ryanodine-sensitive calcium stores, we performed some preliminary measurements of $[Ca^{2+}]_i$ after the addition of 20 mM caffeine.

## 2. Materials and methods

### 2.1. Cell culture

Human astrocytoma cell line U-373 MG cells were obtained by courtesy of Prof. Chieco-Bianchi (Institute of Oncology, University of Padua, Italy). The cells were cultured in DMEM (Seromed, Biochrom, Germany) supplemented with 10% fetal calf serum (Seromed, Biochrom), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cell number was determined by light microscope count and via-

bility was assayed by the trypan blue dye exclusion technique. Dilutions of $1.25\times10^5$ cells/ml were distributed (200 μl/well) in quadruplicate in 96-well culture plates (Costar, Badhoevedorp, The Netherlands) and placed in a 95% air:5% $CO_2$ incubator (KW, Siena, Italy) saturated with $H_2O$ at a temperature of $37\pm0.2$°C, with internal EMF background of $0.5\pm0.2$ μT, probably due to heater and fun.

After 12 h, cells were exposed to an EMF either as above or after stimulation with 1.2 μM substance P (Sigma, St. Louis, MO, USA) immediately before the exposure. This concentration was selected on the basis of preliminary experiments.

Microtitre plates were placed in a rectangular solenoid (exposure chamber) formed by a 2500 turn coil providing a central homogeneous magnetic field. Plates were placed horizontally in an area of the coil where the magnetic field was uniform. The exposure chamber was water jacketed, the water flow being provided by a pump connected to a remote reservoir maintained isothermal with the field exposure samples at $37\pm0.2$°C. The system was powered by a pulse generator (GW Instruments, Hsien Tien City, Taiwan), maintained outside the incubator, which produced in the system a square wave of 50 Hz frequency with rise time of 2.7 ms, a fall time of 2.4 ms and a duty cycle of 1/2. The current in the coil was 120 mA. The system generated a peak magnetic field of 3 mT as measured by a Hall effect probe. Both the normal and substance P-stimulated cultures were exposed to EMFs for 24 h. During this period, no thermal effects, monitored by a thermoresistor with a sensitivity of $\pm0.1$°C placed directly in the microwells, were observed. Sham-exposed cells were put simultaneously into a sham exposure apparatus identical to the experimental apparatus but not energized, where the EMF background remained practically unchanged ($0.6\pm0.5$ μT). Immediately after EMF exposure or sham exposure (0 time) and after incubation in the same incubator for 24 and 48 h, all microwells were centrifuged and the supernatants were collected and frozen at −20°C until determination of IL-6.

### 2.2. Measurement and calculation of $[Ca^{2+}]_i$

Three millilitres of cell suspension containing $2.5\times10^5$ cells/ml, either normal or treated with 1.2 μM substance P, were placed into 35 mm Petri dishes containing two 10 mm diameter circular glass coverslips and incubated at 37°C for 24 h in air:$CO_2$ until confluence was achieved. The cells were then exposed to EMFs for 24 h. At the end of the exposure period, glass coverslips containing adherent cells were removed from the Petri dishes and washed two times with HEPES-buffered saline (HBS) containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose at a pH of 7.4, supplemented with 0.03% Pluronic F-127. In some experiments, to evaluate the provenance of the $[Ca^{2+}]_i$, $CaCl_2$ was substituted with NaCl before the measurement. Then U-373 MG cells were incubated for 75 min at 25°C in the dark with 10 μM fura 2 acetoxymethyl ester (fura 2/AM) in dimethyl sulphoxide and, at the end of the incubation period, were washed three times with HBS. $[Ca^{2+}]_i$ was monitored by single excitation fluorimetry (Shimadzu 5000, Duisburg, Germany); each coverslip was placed in a fluorimetric cuvette containing 2 ml of HBS thermostatted at 30°C, and fluorescence ($F$) was recorded at 340 nm excitation and 505 nm emission. In some experiments the measure was repeated after the addition of 20 mM caffeine. Immediately after the measurements, the calibration was performed on each sample to evaluate $[Ca^{2+}]_i$. Fura 2 leakage was estimated by quenching the extracellular dye fluorescence with 0.2 mM $MnCl_2$, which was then chelated by the addition of 0.5 mM CaDTPA.

The maximal fluorescence ($F_{max}$) was obtained by adding sequentially 10 nM $CaCl_2$, 2.3 μM $Ca^{2+}$ ionophore A 23187 and 100 μM digitonin. Finally, 20 mM $MnCl_2$ was added to record the autofluorescence, from which the minimum fluorescence ($F_{min}$) was calculated according to Hesketh et al. [32]. $F$, $F_{min}$ and $F_{max}$ values were corrected for autofluorescence and $[Ca^{2+}]_i$ was calculated from $F$ according to Tsien et al. [33] and Grynkiewicz et al. [34]. $[Ca^{2+}]_i$ indexes were calculated by division of the $[Ca^{2+}]_i$ nanomoles obtained after EMF exposure by those obtained after sham exposure.

### 2.3. Proliferation assay

The proliferative responses of cells to EMFs were evaluated in quadruplicate on U-373 MG cells by a colorimetric assay [35] after the different periods

of incubation. Briefly, 20 μl of prefiltered [3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide] (MTT, Sigma) solution (5 mg/ml in PBS) were added to each well and the plate was incubated for 3 h at 37°C. Supernatants were then removed and the formazan crystals dissolved by the addition of 100 μl of an isopropanol solution containing 0.04 N HCl. After a few minutes, when all crystals had dissolved, plates were read on a Titertek Multiskan Micro-ELISA Reader (Flow Laboratories, Helsinki, Finland) using a wavelength of 570 nm and a reference wavelength of 630 nm. Proliferation indexes were calculated by division of the absorbance values obtained after EMF exposure by those obtained after sham exposure.

### 2.4. Determination of interleukin-6

IL-6 was determined with immunoenzymatic methods. The commercial enzyme-linked immunosorbent assay was Cytoscreen Human IL-6 (Biosource International, Camarillo, CA, USA). The minimum detectable dose was 2 pg/ml. Samples were used undiluted. A three-cycle automatic washing was routinely performed. Yields ranged between 88 and 105%. Both kit standards and our internal standards were diluted in RPMI 1640 medium. IL-6 production ratios were calculated by division of the experimental values by the control values.

### 2.5. Statistical analysis

$[Ca^{2+}]_i$ was expressed in nanomoles, IL-6 was expressed as pg/ml and both were reported as mean ± S.D. Statistical evaluation of the experimental data was performed with two-tailed Student's *t*-test for paired samples with $P < 0.05$ as the minimum level of significance.

## 3. Results

A significant increase in $[Ca^{2+}]_i$ was observed after 24 h of EMF exposure (Fig. 1a). The basal $[Ca^{2+}]_i$ concentrations increased from 124 ± 51 nM to 200 ± 79 nM and the release indexes, calculated as described in Section 2, rose to 1.7 ± 0.5. There was a significant difference ($P < 0.03$) between the values

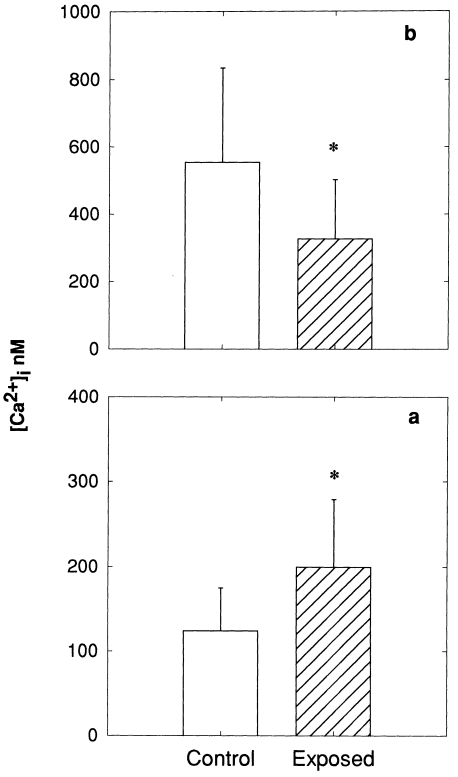


Fig. 1. $[Ca^{2+}]_i$ measured in control and EMF-exposed U-373 MG cells without (a) and after (b) pretreatment with 1.2 μM substance P. $*P < 0.03$, $n = 10$.

(expressed as nanomoles or as release indexes) obtained from exposed cells and those from sham-exposed cells. Preincubation of cells with substance P caused a marked increase in $[Ca^{2+}]_i$ levels in both sham- and EMF-exposed cells (Fig. 1b). However, after exposure there was a modest increase with respect to the $[Ca^{2+}]_i$ in cells without substance P. Thus we observed a significant ($P < 0.004$) decrease in $[Ca^{2+}]_i$ after exposure (Fig. 1b) as compared to sham-exposed cells. This indicated that EMFs could either cause efflux of $Ca^{2+}$ from the cells or its redistribution among intracellular compartments. The typical fluorescence signals from astrocytes are reported in Fig. 2: there was an increase in signal after 24 h of EMF exposure as compared to sham-exposed cells, which increased further after addition of 20 mM caffeine. Finally the combined effects of EMFs and substance P on the decrease in $[Ca^{2+}]_i$ were more clearly demonstrated when $[Ca^{2+}]_i$ measurements were carried out in a $Ca^{2+}$-free medium. In this case, exposure to EMFs dramatically reduced the

$[Ca^{2+}]_i$ (Fig. 3b). In contrast, exposure did not cause a reduction of $[Ca^{2+}]_i$ in cells not stimulated with substance P and the concentrations were practically identical to those measured in medium containing $Ca^{2+}$ (Fig. 3a).

$[Ca^{2+}]_i$ variations after exposure to EMFs were taken as a parameter of the modification of both the proliferation and production of cytokines.

The proliferative responses, although slightly increased 0 and 24 h after the 24 h EMF exposure of U-373 MG cells, were negligible compared with control values, while the proliferation indexes were about 1. Also after pretreatment of cells with substance P, we failed to observe any significant variation of the proliferation indexes (Table 1).

U-373 MG cells, incubated for up to 48 h at 37°C in air:$CO_2$ after 24 h of EMF exposure, spontaneously released about 10 pg/ml of IL-6. Immediately after the EMF exposure period and 24 and 48 h thereafter, the release of IL-6 increased slightly without significant differences between the EMF-exposed and sham-exposed groups (Fig. 4a).

When the cells were stimulated with substance P before the exposure, IL-6 concentrations increased significantly with respect to cells without substance P. Moreover, IL-6 levels, measured immediately after the exposure period in supernatants of both sham-exposed and EMF-exposed cells, were $28.5 \pm 20$ and $43.7 \pm 30.9$ pg respectively and the differences between the two groups were significant. Then the IL-6 levels in the supernatants of exposed cells rose significantly to $50.4 \pm 23.1$ pg 24 h thereafter and remained practically the same during the following 24 h (Fig. 4b). The previous findings were confirmed by comparing the cytokine production ratio between EMF-exposed and unexposed cells, both normal and stimulated with substance P: the results were very similar and more homogeneous.

## 4. Discussion

The main aim of the present study was to evaluate whether astrocytoma cells are in an active state after exposure to EMFs. As many of the in vitro studies using EMFs are hardly reproducible [28], in order to maintain as constant as possible the physical and biological variables, the EMF-exposed and sham-exposed cells were kept at $37 \pm 0.2$°C and treated simultaneously in the same incubator, which had a background of $0.6 \pm 0.5$ μT. Moreover, as data from experiments with tumour cells must be interpreted

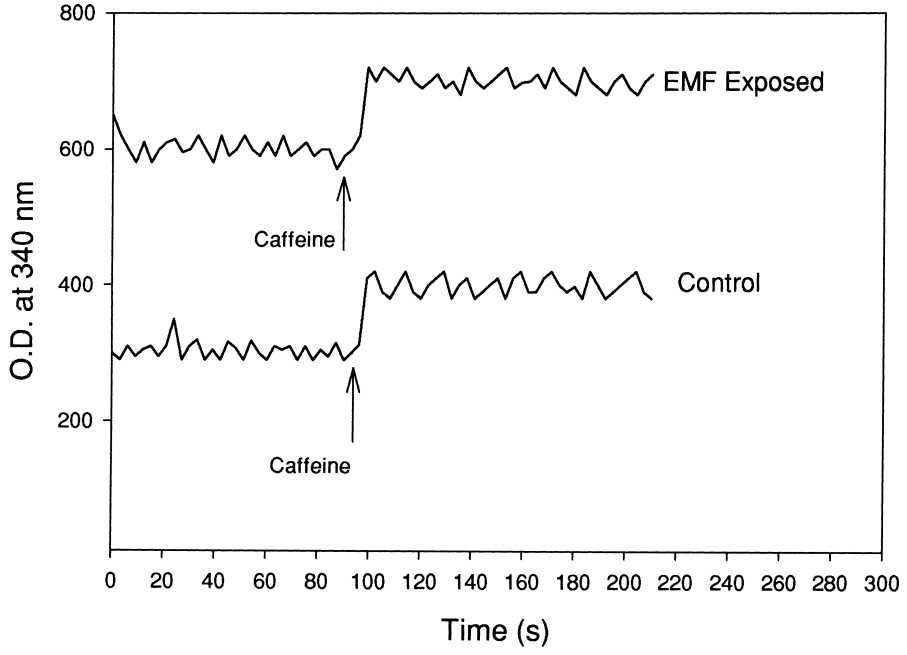


Fig. 2. Typical fluorescence signal from sham-exposed and EMF-exposed cells in normal conditions and after the addition of 20 mM caffeine (↑). The tracing represents two experiments.

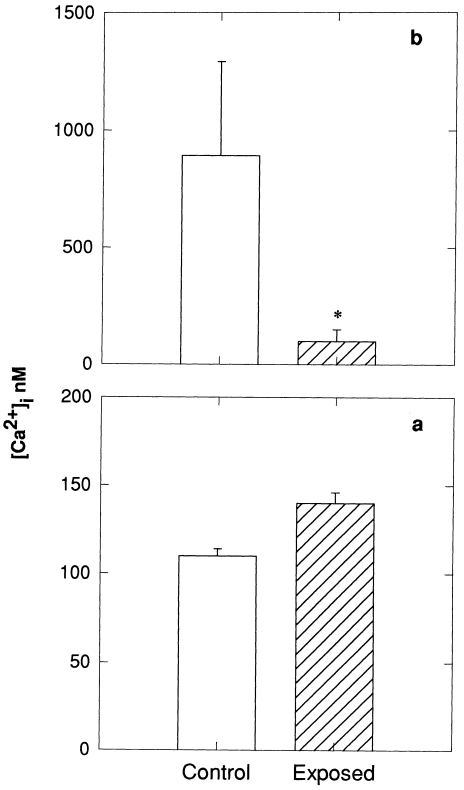


Fig. 3. $[Ca^{2+}]_i$ measured in control and EMF-exposed U-373 MG cells without (a) and after (b) pretreatment with 1.2 μM substance P after substitution of normal medium with $Ca^{2+}$-free medium. $^*P < 0.05$, $n = 3$.

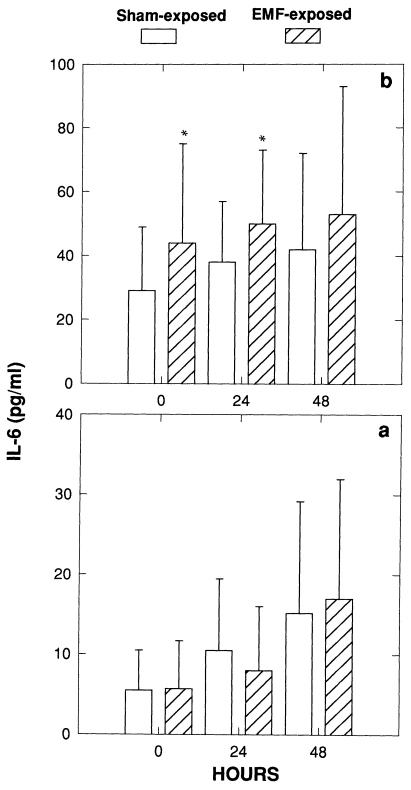


Fig. 4. Interleukin-6 (pg/ml) produced by normal (a) and substance P-pretreated cells (b) during 48 h of incubation at 37°C after EMF exposure or sham exposure (0 time represents the end of the 24 h exposure period). $^*P < 0.05$, $n = 10$.

with caution, we used U-373 MG cells accordingly with the results of others which indicated that data obtained using astrocytoma cell lines are well comparable with those obtained with primary astrocytes [24]. It is known that EMFs are unable to transfer a significant amount of energy to cells or to modify the electrical potential of the plasma membrane, although they can alter its structure and function [3,36]. Since $[Ca^{2+}]_i$ has a crucial role in the regulation of the cell cycle [37], we evaluated the movements of this ion after EMF exposure in normal cells and in cells pretreated with substance P. Our results indicate that $[Ca^{2+}]_i$ is rapidly but not transiently elevated after EMF exposure of normal U-373 MG cells; the findings agree well with those of Morgado-Valle in PC12 cells [27].

Table 1
Proliferation indexes of both normal and substance P-stimulated U-373 MG cells (after incubation at 37°C) evaluated at different times after EMF exposure

| Time after exposure (h) | Without substance P | With substance P |
|---|---|---|
| 0 | 1.03 ± 0.17 | 1.04 ± 0.09 |
| 24 | 1.07 ± 0.09 | 1.06 ± 0.06 |
| 48 | 1.00 ± 0.07 | 0.99 ± 0.12 |

$n = 10$.

At millimolar concentrations, caffeine is a high affinity ligand for the $Ca^{2+}$-induced $Ca^{2+}$ release channel, and it triggers $Ca^{2+}$ release when intracellular stores contain $Ca^{2+}$ [38]. Although preliminary, our results indicate the absence of any synergy between EMF exposure and caffeine in increasing $[Ca^{2+}]_i$ concentrations. In fact the per cent increase after the addition of caffeine was the same in sham-exposed and EMF-exposed cells. Activation of NK-1 receptors by substance P is responsible for a variety of responses, including increases of phosphoinositides and intracellular calcium [24]. Moreover, $Ca^{2+}$ release channels, not only in the endoplasmic reticulum but also in other organelles, can be activated by

either inositol triphosphate receptors or caffeine-ryanodine receptors [39]. Thus the incubation of cells with 1.2 μM substance P caused a marked increase in $[Ca^{2+}]_i$. In this case, EMF exposure was responsible for a significant decrease in $[Ca^{2+}]_i$ with respect to control cells. Evaluation of $[Ca^{2+}]_i$ in cells incubated in calcium-free medium revealed about 1 μmole of $[Ca^{2+}]_i$, which was dramatically reduced to about 100 nmoles after EMF exposure. Taken together, our results suggest that, by changing the properties of cell membranes, EMFs can influence $Ca^{2+}$ transport processes and hence $Ca^{2+}$ homeostasis. The processes involved may include both the ATPase-dependent $Ca^{2+}$ pumps present in endoplasmic reticulum and in the plasma membrane, the $Na^+/Ca^{2+}$ electroneutral exchanger and the VOC-dependent $Ca^{2+}$ channels. Even though the mechanism involved remains to be elucidated, our data suggest that the overall effect of EMFs might depend on the prevailing $Ca^{2+}$ conditions of the cells. Thus, whereas with normal $[Ca^{2+}]_i$ EMFs seem to stimulate the influx of extracellular $Ca^{2+}$ with a consequent increase of intracellular levels, under high $[Ca^{2+}]_i$ (as induced by pretreatment with substance P) EMF exposure seems to stimulate $Ca^{2+}$ efflux or alternatively a redistribution of the ion among intracellular compartments. The latter effect seems to depend on the $[Ca^{2+}]_i$ gradient between intra- and extracellular compartments, since it was enhanced when measurements were carried out in $Ca^{2+}$-free medium. Although this corroborates our hypothesis about the involvement of EMFs in $Ca^{2+}$ efflux, it also indicates that a simple passive diffusion of the ion from the cells may be part of the mechanism mediating the $Ca^{2+}$ response to EMFs in substance P-pretreated cells. Moreover, since this effect is long lasting, being present even 1 h after exposure (data not shown), a transient effect of EMFs after 24 h of exposure must be excluded.

Since calcium movements are responsible for cell proliferation and neurite growth [27,37] we evaluated the proliferation of U-373 MG cells after 24 h of EMF exposure. Our results clearly indicate the absence of any spontaneous proliferation of normal and substance P-pretreated cells after EMF exposure.

One effect of EMF exposure is stimulation of the stress response, which is known to be initiated by many stimuli, including oxidative injury, free radicals and chemicals [40]; it is also well known that cells produce cytokines after treatment with agents increasing their intracellular calcium concentration [41]. Cytokines have been shown to mediate the response of the central nervous system to mechanical injury; in particular, it has been shown that traumatic injury induces IL-6 release by human astrocytes in direct relation to the severity of the barotrauma [42]. In fact, in physiological conditions, IL-1 and IL-6 are located in neurones and glial cells of discrete brain regions and are expressed at a constant level during peripheral immune challenge. After severe brain damage both the synthesis and release are increased. We chose to evaluate IL-6 since its level is a true indication of the magnitude of inflammatory and stress responses. As demonstrated by our results, although EMF exposure alone increased $[Ca^{2+}]_i$, it was unable to induce high levels of IL-6. Leal-Berumen et al. [41] demonstrated that peritoneal mast cells stimulated with Ca ionophore A23187 were unable to produce IL-6. After substance P challenge the levels of IL-6 rose significantly upon EMF exposure. The greatest effects were obtained immediately after the exposure period and 24 h thereafter. EMFs probably exert a modulatory effect on cells which is strengthened by substance P stimulation [43], this kind of 'priming' effect being required for the U-373 cells to enter a state of responsiveness to EMFs [44].

On the whole, our results clearly demonstrate movements of calcium after exposure to EMFs and that astrocytoma cells subjected to pulsed EMFs for 24 h were unable to modify their spontaneous proliferation rate, although $[Ca^{2+}]_i$ was increased after exposure. The concentration of IL-6 increased slightly during the incubation period without any significant difference between EMF-exposed and sham-exposed cells. Exposure of U-373 MG cells after pretreatment with substance P caused significant increases of IL-6. Finally, for an understanding of the role of $Ca^{2+}$ stores and plasma membrane L-channels in the $Ca^{2+}$ movements after EMF exposure, experiments using blockers of L-channels (dihydropyridines, phenylalkylamines and benzothiazepines) and agonists (cyclic ADP-ribose) and antagonists (rutenium red, thapsigargin) of the ryanodine-sensitive and $IP_3$-sensitive $Ca^{2+}$ stores are necessary.

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